BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Am J Physiol Gastrointest Liver Physiol 282: G735–G741, 2002.
First published January 9, 2002; 10.1152/ajpgi.00510.2001.

themes

Cutting-Edge Technology II. Proteomics: core technologies and applications in physiology

FRANK A. WITZMANN AND JUNYU LI
Departments of Cellular and Integrative Physiology and Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Witzmann, Frank A., and Junyu Li. Cutting-Edge Technology. II. Proteomics: core technologies and applications in physiology. *Am J Physiol Gastrointest Liver Physiol* 282: G735–G741, 2002. First published January 9, 2002; 10.1152/ajpgi.00510.2001.—Technologies for proteomics, e.g., studies examining the protein complement of the genome, have been in development for over 20 years. More recently, proteomics has become formalized by combining techniques for large-scale protein separation with very precise, high-fidelity approaches that analyze, identify, and characterize the separated proteins. These methods bring to reality the powerful scope of proteomics, enabling researchers to investigate cellular function at the protein level and thus representing one of proteomics' most fitting applications. In this review, we take a brief and concise look at some of the current, physiologically relevant technologies that comprise proteomics and report specific applications in which proteomics has provided valuable biological insight.

protein analysis; two-dimensional electrophoresis; mass spectrometry; isotope-coded affinity tags

THE BROAD RANGE OF MOLECULAR mechanisms that governs cellular function is largely administered via the structure and function of genetically encoded products, the proteins. Collectively, these gene products represent the proteome, and their analysis has come to be known as proteomics. The actual number of functionally unique protein types in the human proteome variably expressed across assorted human cell types from the >30,000 available genes is estimated to be 100,000. With multiply-modified forms of each, that number could approach a million. This diversity is the result of widespread posttranscriptional processing of mRNA and co- and posttranslational processes. Both of these lead to a fair degree of discordance between the open reading frames predicting protein structure and the actual functional product. Consequently, a full understanding of function, disease processes, and clinical intervention necessitates expression analysis at the protein level. Additionally, the range of fully functional protein abundance in a cell may reach nine orders of magnitude. Proteomics thus presents investigators with a daunting technological task, both in terms of protein identification and quantification. Although originally designated as a global approach to identify the entire proteome (34, 36), using two-dimensional (2D) electrophoretic (2DE), mass spectrometric, and bioinformatic techniques, proteomics has become a diverse science that includes nearly all manner of separation, affinity purification, and protein chemistry components.

Considerable effort has been and continues to be placed on removing the technical barriers that impede proteomic efforts. We now understand both the strengths and limitations of the "first generation" proteomics approaches capable of generating significant biological insight, yet generally providing narrow data (protein presence/absence, protein identification) for high and moderately abundant proteins (6). This realization has led to expanded development and implementation of chromatographic separation techniques, improved mass spectrometry (MS), automation via robotics, and growth of multidimensional biomolecular datasets (e.g., posttranslational modifications, subcellular localization, protein interactions, protein abundance, and protein function). Further technological developments will continue to drive forward the next generation of proteomic techniques and approaches. These developments have widened the scope of proteomics and have fueled the explosion of interest in this field. Two full issues of *Trends in Biotechnology* have addressed these developments in detail (4, 37), and readers are encouraged to consult them. This themes article highlights some of the technologies central to contemporary proteome analysis and provides

Address for reprint requests and other correspondence: F. A. Witzmann, Depts. of Cellular and Integrative Physiology and Biochemistry and Molecular Biology, Indiana Univ. School of Medicine, 635 Barnhill Dr., MS405, Indianapolis, IN 46202 (E-mail: fwitzman@iupui.edu).

examples of how these have been applied to physiological questions.

DIFFERENTIAL EXPRESSION PROTEOMICS

2DE and MS. As mentioned above, proteomics originated as a direct result of technical developments in 2DE protein separation and MS instrumentation and the explosion of genome sequence information that generated protein sequence databases. Often referred to as "peptide mass fingerprinting," this first-generation, or "blue-collar," proteomics approach is still the most commonly used proteomics strategy and the most practical and economical for academic laboratories.

In 2DE, proteins are subjected to orthogonal separation methods; the first based on protein charge via isoelectric focusing (IEF) and then by mass in sodium dodecyl sulfate PAGE. The relatively recent development of immobilized pH-gradient gel (IPG) strips to improve first-dimension IEF separations shows promise, although gel-based IEF remains a useful tool for the patient and resourceful. The final product of 2DE separation is essentially an in-gel array of proteins, each assuming a coordinate position corresponding to the unique combination of isoelectric point (pI) and mass. Resulting 2D protein patterns are visualized by a number of methods: visible and/or fluorescent dyes, silver stains, or autoradiography. Typically, scanned gel images are analyzed by any of a number of ever-improving 2D gel analysis software packages. It is here that both the strengths and weakness of this approach become evident. Protein abundance comparisons (e.g., differential expression) are easily made, because differences in protein spot density are readily detectable and can be quantified robustly and compared statistically. However, unless one conducts highly parallel 2DE runs, gel-to-gel variation becomes problematic and image analysis an exercise in frustration.

Despite the insightful design and implementation of parallel 2DE nearly 24 years ago (2, 3) and numerous examples of its utility in differential protein expression analyses across a large number of samples, surprisingly, this approach has not been used widely. Unlike trends in 2D gel analysis software that enable the concurrent analysis of hundreds of gel patterns per experiment, electrophoretic equipment manufacturers have lagged behind. Although efforts have been made to address the technical necessity of highly parallel 2DE by scaling the process up to 12 gels/run maximum (e.g., Bio-Rad, Amersham Biosciences, etc.), contemporary 2DE instrumentation still falls short of the scale necessary (>20–24 2D gels/run). Figure 1 illustrates this point by presenting a montage of multiple patterns from a single 2DE experiment. Here, 36 individual 2D gels were run (20/run) analyzing 36 individual wells from six 6-well culture plates on which human keratinocytes were cultured. Parallel analysis of this type

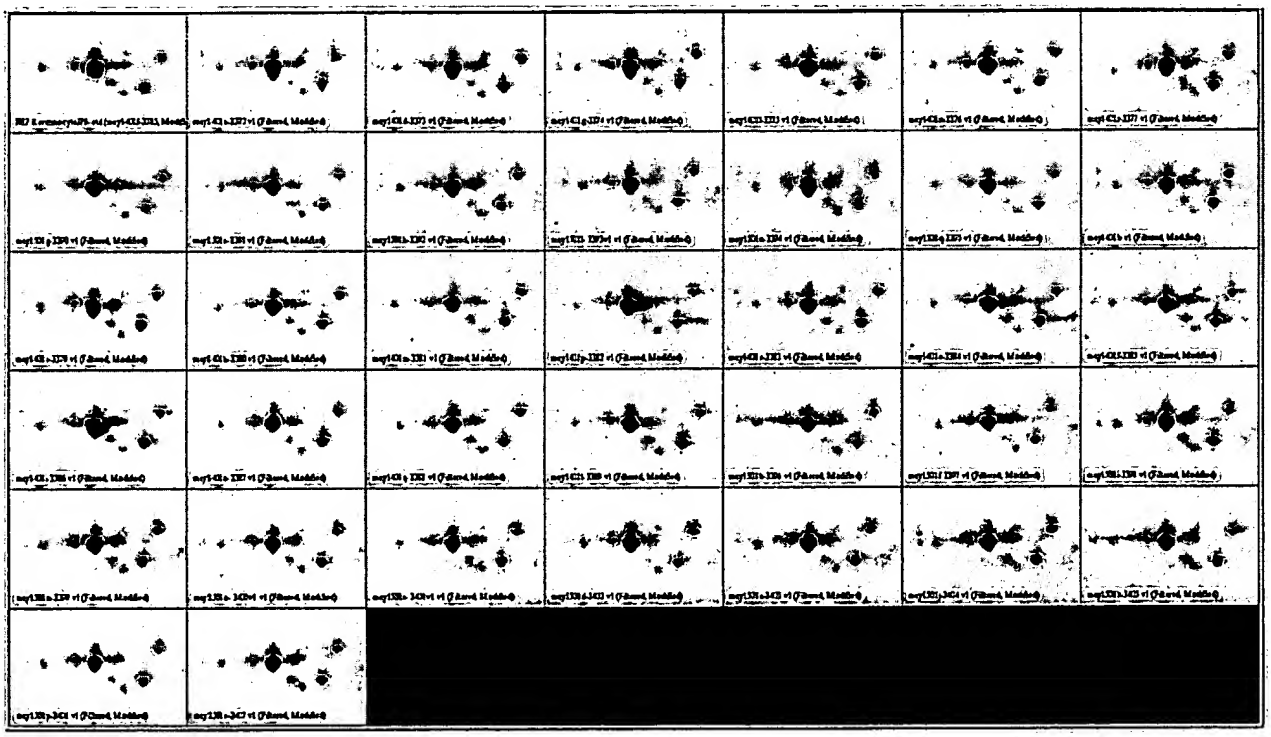

Fig. 1. Example of gel-to-gel consistency across 36 individual gel patterns representing 36 individual human keratinocyte samples incubated in six 6-well plates and solubilized on the plates (medium removed). Samples were separated by 2-dimensional (2D) electrophoresis (2DE), 20 gels/run in the authors' laboratory; thus results from 2 separate runs are shown. The first (*top left*) pattern is the reference pattern in PDQuest analysis, the next 12 patterns are controls, and the remaining 24 are jet fuel exposed. Highly parallel 2DE is essential for inclusion in successful differential expression proteomics studies.

makes differential expression analysis robust and simplifies candidate protein selection.

A recent development that addresses gel-to-gel variability using 2DE incorporates sensitive fluorescent protein staining and can be achieved without extensive parallel 2DE instrumentation is 2D differential gel electrophoresis (DIGE) (32). By using replicate gels of pooled samples separated on single 2D gels from multiple individual animals (control vs. treated in each gel) (31), this approach has been used and validated to determine quantitative protein differences in acetaminophen toxicity. In this clever approach, control and treated samples are labeled with Cy3 or Cy5 dyes and mixed before application onto the same 2D gel. As a result, the same form of a given protein from each sample will migrate to the same position on the 2D gel. The relative abundance of each protein in each sample is then obtained by scanning the gel using excitation and emission wavelengths unique to each Cy dye.

After 2DE separation and image analysis, any or all of the proteins in the gel pattern can be selected for identification. In large, 2D gel-based projects, the ultimate goal is to identify all resolved proteins. In less ambitious studies, only those proteins differentially expressed are of interest. Regardless of project scope, the general approach to protein identification is to cut the protein spot from the gel and digest it with a proteolytic enzyme such as trypsin. The resulting digest mixture is then analyzed by matrix-assisted laser desorption ionization (MALDI) time of flight MS (26). The measured and optimized monisotopic mass data are then compared with theoretically derived peptide mass databases, generated by applying specific enzymatic cleavage rules to predicted/known protein sequences. Whereas MALDI-based peptide mass fingerprinting enables high-throughput, accurate, and sensitive mass detection and may result in a large percentage of convincing protein identifications, many are ambiguous and require confirmation. Frequently, some digested proteins remain completely unidentified, despite yielding measurable peptides.

For unambiguous identification of 2D separated proteins, "peptide sequence tag" data derived by MS/MS (27) (with either MALDI or electrospray ionization ion sources) can be compared with expressed sequence tag databases, ever-expanding sources of genomic/proteomic information representing a number of organisms.

Because the dynamic range of protein expression in most whole cell or tissue lysates is huge and only the most abundant proteins from 2D gels can be analyzed (despite excellent mass spectrometer sensitivity), far too many proteins are overlooked. Furthermore, hydrophobic proteins and those with very alkaline pI are poorly resolved on conventional 2D gels. Even in rarely employed very large format gels, no more than 10,000 proteins have been analyzed on a single gel. To overcome the problems of sensitivity and scope, tissue/cell fractionation methods are used to enrich the sample gels with organellar proteins, and as a result, several organellar proteomes are being characterized (9, 15, 19, 30).

Narrow-range IPG strips in 7-, 11-, 17-, or 24-cm lengths can bracket the pH range of first-dimension separations extending to fairly alkaline pH. Using these strips represents another significant step in overcoming the limits of 2DE by significantly expanding the resolving power of otherwise broad-range IEF. Separations achieved in narrow pI windows greatly increase resolution and provide access to proteins that are either undetectable or comigrate when separated using a broad-range pH gradient. This approach thus becomes integral to any attempt to analyze thousands of sample proteins, particularly for those low-abundance proteins that would otherwise remain undetected. In this regard, "virtual" gel patterns, such as those described by Cordwell et al. (5), significantly increase the number of proteins resolved (Fig. 2) and represent an important technical development. Finally, significant improvements in protein-detection sensitivity have been achieved by incorporating fluorescent and MS-compatible silver stains (22, 24, 29). In combination, these technical advances continue to make 2DE an important component of the proteomics arsenal.

For example, 2DE, fluorescent staining (2D-DIGE), image analysis, and peptide mass fingerprinting were combined recently to analyze the cardiac mitochondrial proteome in murine creatine kinase (CK) double-knockouts (KO) (20). Proteomic analysis demonstrated that despite the absence of all isoforms of CK in the KO mice, the cardiac mitochondrial proteome was identical to wild types, and, more importantly, its consistency mirrored a lack of altered cardiac function. Similarly, this approach was used recently to study human bronchial biopsy samples. Proteins from these samples, which correlated to the transformation of normal fibroblasts to myofibroblasts, were quantified and identified during the remodeling processes observed in asthma (35). Large-scale protein database development for toxicologic/pharmacological applications is an ongoing enterprise in many industrial laboratories. This is typified by Fountoulakis' 2D gel-based mouse liver protein database (8) that lists hundreds of identified proteins screened against acetaminophen and cites numerous other databases designed for general toxicologic screening applications.

Isotope-coded affinity tags. An emerging approach that directly addresses the dynamic range and solubility limitations of 2DE combines the separating power of liquid chromatography (LC) with the highly accurate and sensitive mass detection of tandem MS (13). Isotope-coded affinity tags (ICATs) are reagents containing a cysteine-reactive group, a linker with either eight hydrogens (light) or deuteriums (heavy), and a biotin (affinity) moiety. As shown in Fig. 3, by reacting each with light or heavy ICAT, relative protein abundance comparisons between two different cell states can be made. The proteins from each sample are combined and proteolytically digested, tagged peptides are collected by affinity chromatography, peptides are analyzed via LC-MS for relative quantitation of the iso-

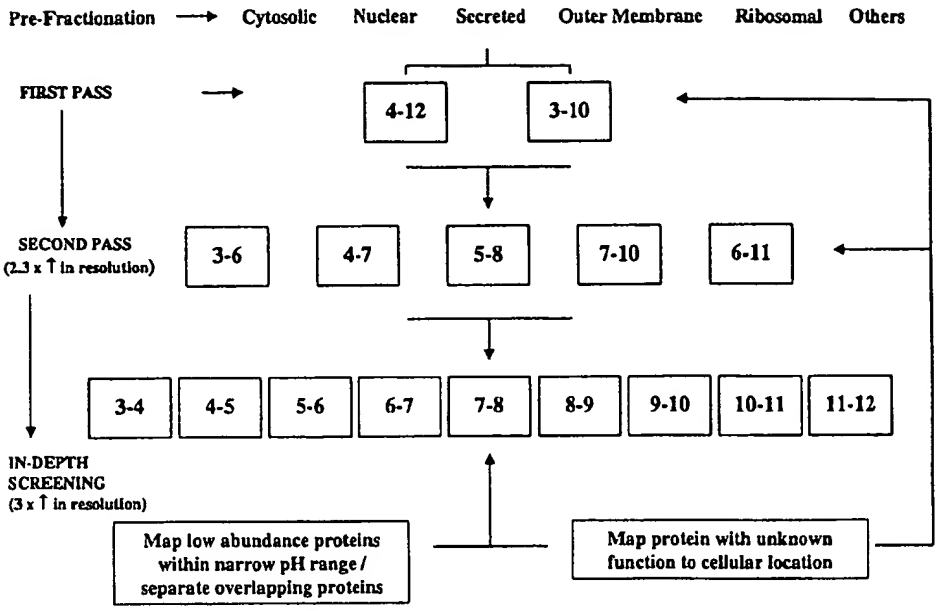

Fig. 2. Schematic view of subproteome approach. Cells and tissues are initially prefractionated into cellular compartments or relative protein solubilities. The samples are screened using wide-range 2D gels to determine sample complexity and then, if necessary, passed through higher resolution 2D to map low-abundance proteins, efficiently separate overlapping proteins, and to map proteins to their cellular location. Modified from Ref. 5.

topes on identical peptides, and finally, peptides are analyzed by LC-MS/MS for protein identification. Despite limitations of its own, ICAT technology is being improved (28) and has already proven its utility in functional studies. For example, characterization of analytically troublesome lipid-raft proteins has been simplified (33), the proteomic components of a complex cellular metabolic pathway have been studied in the context of their functional genomic elements (17), and proteins of subcellular microsomes have been identified and quantified in differentiating human myeloid leukemia (HL-60) cells (14) using the ICAT approach. The latter investigation included a common addition to contemporary proteomic approaches, e.g., multidimensional chromatographic separation of complex peptide mixtures. In this case, sample complexity was reduced by subjecting isotopically labeled proteolytic peptide mixtures to cation-exchange chromatography, avidin-affinity chromatography and reversed-phase HPLC before automated mass spectrometric characterization.

Because an estimated 20% of the human proteome includes proteins lacking at least one cysteine residue, alternatives to cysteine-based labeling that can tag every protein are being developed. For instance, a clever variation of the ICAT approach has been developed to identify and quantitate the extent of protein

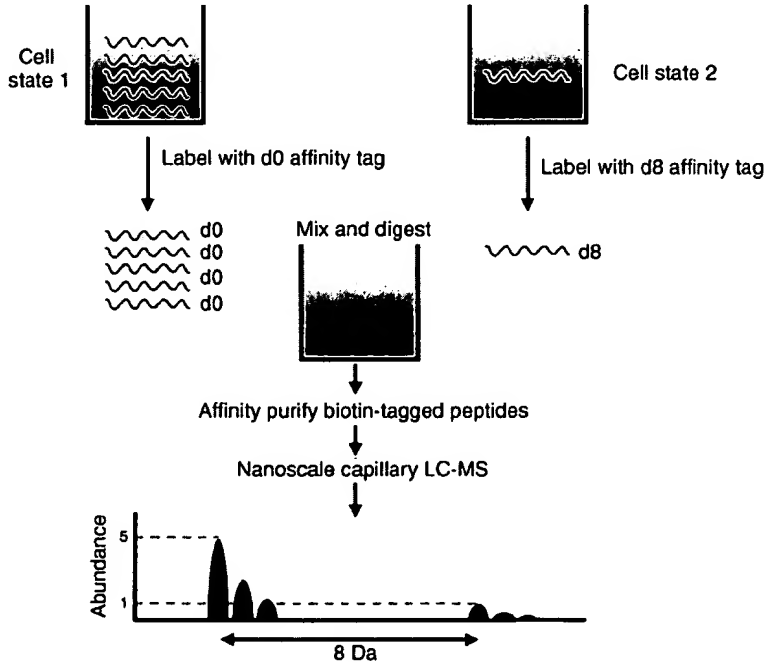

Fig. 3. Schematic representation of the isotope-coded affinity tag method. The cysteine side chains in the complex mixtures of proteins from 2 different cell states are reduced and alkylated using the *day 0* (d0)-labeled tag for the proteins in 1 cell state and the *day 8* (d8) form of the tag for the proteins in the second cell state. The 2 mixtures are then combined and subjected to a proteolytic digestion. The resultant complex mixture of proteolytic peptides is purified with an avidin column to pull out only the subset of labeled peptides via the affinity tag. Quantitation of differential expression is based on the relative abundance of the isotopes in the mass spectrometry spectrum. Modified from Mosely MA, *Trends Biochem Sci, Suppl* 19, S11, 2001.

phosphorylation using a phosphoprotein isotope-coded affinity tag (PhIAT) (12). The PhIAT methodology is similar to the ICAT approach in that it enables proteome-wide purification and quantitation of peptides containing specific types of residues; in this case phosphopeptides. Its potential in the characterization of cellular signaling is promising.

To both simplify complex peptide mixtures mentioned above and to target specific subgroups of related proteins and selectively identify them, a strategy using "signature peptides" (10, 18) isolated by an array of elaborate yet automated affinity and chromatography separations is being developed to study global phospho- and glycoprotein expression (11, 25). These multidimensional separation approaches represent an effective trend in proteomics (independent of ICATs), and their implementation should prove particularly useful in physiologically directed differential expression and qualitative proteomics studies.

FUNCTIONAL AND STRUCTURAL PROTEOMICS

Many protein-mediated cellular functions are managed and regulated by mechanisms that do not involve quantitative changes in expression. Instead, they are the consequences of qualitative modification of existing proteins, chemical additions such as phosphorylation, glycosylation, and lipidation, or modifications such as oxidation and deamidation. Second, proteins that mediate most cellular processes function as constituents of macromolecular complexes, not as individual entities acting independently. The proteomic approaches described thus far are rather reductionist. Clearly, these are useful ways in which to study the proteome; however, to effectively study function, investigators also must focus on protein-protein interactions and the characterization of multimeric protein complexes. In this respect, two areas in which proteomics is playing a significant role in our understanding of cellular function are the characterization of posttranslational modifications (functional proteomics) and protein interactions (structural proteomics).

The phosphoproteome. Given the importance of protein phosphorylation in the regulatory activities of cellular function and the amplification of signaling cascades that distinguish these activities from others, it is not surprising that phosphorylation is the most common covalent protein modification in mammalian cells. Indeed, the huge number of protein kinases and phosphatases encoded by the genome underscore their significance. Global analysis of the phosphoproteome has thus evolved into an integral facet of physiology. Historically, phosphoproteins were studied on Western blots using antiphosphoserine or antiphosphothreonine antibodies. This approach is still adequate qualitatively but not quantitatively, because it suffers from the same general limitations of 2DE mentioned earlier. In-gel digestion and phosphopeptide analysis are deemed feasible but impractical (21). As alternatives, recent analytical approaches to the phosphoproteome incorporate either phosphopeptide enrichment using metal affinity columns, phosphatase treatment before MS/MS, or the use of protein chips (39). These approaches are necessitated by the low stoichiometry of protein phosphorylation, the fact that phosphopeptides are generally detected with low efficiency or not at all by MS. Also, the hydrophilic phosphopeptides may be eluted and therefore lost in the void volume during reversed-phase peptide cleanup for MALDI.

New methods are in use that combine chemical modification and affinity purification for the characterization of serine and threonine phosphopeptides (1, 23). These methods are generally based on the chemical replacement of the phosphate moieties by affinity tags (biotinylation) followed by trypsin digestion. The biotinylated peptides are then enriched by affinity-isolation, analyzed by LC-MS/MS, and the phosphorylated residues are identified by automated database searching. This approach has widespread potential utility for defining signaling pathways and control mechanisms that involve phosphorylation or dephosphorylation of serine/threonine residues.

In a related development, Snyder and his colleagues (40) have engineered a novel approach for high throughput screening of protein kinase (PK) activities by overproducing all the yeast PKs as glutathione *S*-transferase fusions and covalently affixing them to a chip surface in microarray format.

With the use of [^{33}P]ATP, it was discovered that particular proteins are preferred substrates for particular PKs and that many PKs prefer particular substrates. This approach has enormous potential application in the study of mammalian and human PK systems.

Protein-protein interactions. As Eisenberg et al. (7) has so aptly proposed, "a protein is defined as an element in the network of its interactions," and, as such, each protein in living cells functions as part of an extended web of interacting molecules. In this regard, a more holistic (as opposed to global) analysis of the proteome incorporates ingenious approaches that involve *1*) affinity purification of protein complexes, the electrophoretic separation of the components, their tryptic digestion, and the identification of each element (16) or *2*) centrifugation purification of cell components, tryptic digestion of the protein constituents followed by multidimensional liquid chromatography and tandem mass spectrometric identification (38).

As an example of the first approach, Blackstock's group (16) isolated the mouse brain *N*-methyl-D-aspartate (NMDA) receptor multiprotein complex (NRC) and, by analyzing its components, provided information that strongly suggests that subsets of neurotransmitter receptors, cell-adhesion proteins, adapters, second messengers, and cytoskeletal proteins are all organized together into a physical unit comprising the signaling pathway. Furthermore, several novel features of the NRC observed in this study provide valuable insight into the physiological context of NMDA receptor-dependent synaptic plasticity.

In the second approach, over 100 proteins can be analyzed per run via direct analysis of large protein complexes. Applied to the eukaryotic ribosomal pro-

teome, its constituent complex of ~80 unique proteins is rapidly and sensitively characterized, and unique features are identified. This process demonstrates considerable potential in characterizing, as well as detecting alterations in, other functionally relevant protein complexes in a variety of cell systems.

In summary, the various cellular proteomes are dynamic, and fluctuations in their characteristic expression are central to their role in physiological regulation, disease and injury, and their response to chemical intervention. Without a doubt, it is therefore essential that we conduct both broad and directed analyses of the proteome's individual protein components to understand the molecular underpinnings of physiological function. We must work to make certain the technologies supporting such analyses continue to improve, in turn, to ensure that the boundaries to our understanding disappear as a result. Despite the limitations of current proteomics technology, there exist a number of approaches from which to choose, specific for each application. Whether one is interested in the differential expression of a protein or group of proteins that underlie functional alterations, posttranslational modification of resident proteins, or the complex constituency and function of huge multiprotein complexes, the tools are available, and they are improving.

This review has presented a limited sample of the many proteomic approaches and technologies relevant to the physiologist. A cursory look at the published literature by the reader will quickly demonstrate the utility of this approach in life science and the breadth in which its analytical power has been and will continue to be applied.

REFERENCES

1. **Adamczyk M, Gebler JC, and Wu J.** Selective analysis of phosphopeptides within a protein mixture by chemical modification, reversible biotinylation and mass spectrometry. *Rapid Commun Mass Spectrom* 15: 1481–1488, 2001.
2. **Anderson NG and Anderson NL.** Analytical techniques for cell fractions. XXI. Two-dimensional analysis of serum and tissue proteins: multiple isoelectric focusing. *Anal Biochem* 85: 331–340, 1978.
3. **Anderson NL and Anderson NG.** Analytical techniques for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: multiple gradient-slab gel electrophoresis. *Anal Biochem* 85: 341–354, 1978.
4. **Blackstock W and Mann M.** A boundless future for proteomics? *Trends Biotechnol, Suppl* 19: S1–S2, 2001.
5. **Cordwell SJ, Nouwens AS, Verrills NM, Basseal DJ, and Walsh BJ.** Subproteomics based upon protein cellular location and relative solubilities in conjunction with composite two-dimensional electrophoresis gels. *Electrophoresis* 21: 1094–1103, 2000.
6. **Corthals GL, Wasinger VC, Hochstrasser DF, and Sanchez JC.** The dynamic range of protein expression: a challenge for proteomic research. *Electrophoresis* 21: 1104–1115, 2000.
7. **Eisenberg D, Marcotte EM, Xenarios I, and Yeates TO.** Protein function in the post-genomic era. *Nature* 405: 823–826, 2000.
8. **Fountoulakis M, Berndt P, Boelsterli UA, Crameri F, Winter M, Albertini S, and Suter L.** Two-dimensional database of mouse liver proteins: changes in hepatic protein levels following treatment with acetaminophen or its nontoxic regioisomer 3-acetamidophenol. *Electrophoresis* 21: 2148–2161, 2000.
9. **Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, and Desjardins M.** The phagosome proteome: insight into phagosome functions. *J Cell Biol* 152: 165–180, 2001.
10. **Geng M, Ji J, and Regnier FE.** Signature-peptide approach to detecting proteins in complex mixtures. *J Chromatogr A* 870: 295–313, 2000.
11. **Geng M, Zhang X, Bina M, and Regnier F.** Proteomics of glycoproteins based on affinity selection of glycopeptides from tryptic digests. *J Chromatogr B Biomed Sci Appl* 752: 293–306, 2001.
12. **Goshe MB, Conrads TP, Panisko EA, Angell NH, Veenstra TD, and Smith RD.** Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal Chem* 73: 2578–2586, 2001.
13. **Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R.** Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999, 1999.
14. **Han DK, Eng J, Zhou H, and Aebersold R.** Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat Biotechnol* 19: 946–951, 2001.
15. **Hanson BJ, Schulenberg B, Patton WF, and Capaldi RA.** A novel subfractionation approach for mitochondrial proteins: a three-dimensional mitochondrial proteome map. *Electrophoresis* 22: 950–959, 2001.
16. **Husi H, Ward MA, Choudhary JS, Blackstock WP, and Grant SG.** Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat Neurosci* 3: 661–669, 2000.
17. **Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, Bumgarner R, Goodlett DR, Aebersold R, and Hood L.** Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* 292: 929–934, 2001.
18. **Ji J, Chakraborty A, Geng M, Zhang X, Amini A, Bina M, and Regnier F.** Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. *J Chromatogr B Biomed Sci Appl* 745: 197–210, 2000.
19. **Jung E, Heller M, Sanchez JC, and Hochstrasser DF.** Proteomics meets cell biology: the establishment of subcellular proteomes. *Electrophoresis* 21: 3369–3377, 2000.
20. **Kernec F, Unlu M, Labeikovsky W, Minden JS, and Koretsky AP.** Changes in the mitochondrial proteome from mouse hearts deficient in creatine kinase. *Physiol Genomics* 6: 117–128, 2001.
21. **Larsen MR, Sorensen GL, Fey SJ, Larsen PM, and Roepstorff P.** Phospho-proteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics* 1: 223–238, 2001.
22. **Lauber WM, Carroll JA, Dufield DR, Kiesel JR, Radabaugh MR, and Malone JP.** Mass spectrometry compatibility of two-dimensional gel protein stains. *Electrophoresis* 22: 906–918, 2001.
23. **Oda Y, Nagasu T, and Chait BT.** Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat Biotechnol* 19: 379–382, 2001.
24. **Patton WF.** A thousand points of light: the application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. *Electrophoresis* 21: 1123–1144, 2000.
25. **Riggs L, Sioma C, and Regnier FE.** Automated signature peptide approach for proteomics. *J Chromatogr A* 924: 359–368, 2001.
26. **Scheler C, Lamer S, Pan Z, Li XP, Salnikow J, and Jungblut P.** Peptide mass fingerprint sequence coverage from differently stained proteins on two-dimensional electrophoresis patterns by matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS). *Electrophoresis* 19: 918–927, 1998.
27. **Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Boucherie H, and Mann M.** Linking genome and proteome by mass spectrometry: large-scale

identification of yeast proteins from two dimensional gels. *Proc Natl Acad Sci USA* 93: 14440–14445, 1996.

28. **Smolka MB, Zhou H, Purkayastha S, and Aebersold R.** Optimization of the isotope-coded affinity tag-labeling procedure for quantitative proteome analysis. *Anal Biochem* 297: 25–31, 2001.
29. **Steinberg TH, Pretty On Top K, Berggren KN, Kemper C, Jones L, Diwu Z, Haugland RP, and Patton WF.** Rapid and simple single nanogram detection of glycoproteins in polyacrylamide gels and on electroblots. *Proteomics* 1: 841–855, 2001.
30. **Taylor RS, Wu CC, Hays LG, Eng JK, Yates JR 3rd, and Howell KE.** Proteomics of rat liver Golgi complex: minor proteins are identified through sequential fractionation. *Electrophoresis* 21: 3441–3459, 2000.
31. **Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, and Davison M.** Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* 1: 377–396, 2001.
32. **Unlu M.** Difference gel electrophoresis. *Biochem Soc Trans* 27: 547–549, 1999.
33. **Von Haller PD, Donohoe S, Goodlett DR, Aebersold R, and Watts JD.** Mass spectrometric characterization of proteins extracted from Jurkat T cell detergent-resistant membrane domains. *Proteomics* 1: 1010–1021, 2001.
34. **Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, and Humphery-Smith I.** Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis* 16: 1090–1094, 1995.
35. **Westergren-Thorsson G, Malmstrom J, and Marko-Varga G.** Proteomics–the protein expression technology to study connective tissue biology. *J Pharm Biomed Anal* 24: 815–824, 2001.
36. **Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, and Williams KL.** Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev* 13: 19–50, 1996.
37. **Wood R.** *Introduction. Proteomics: A Trends Guide 2000*, Oxford, UK: Elsevier, 2000, p. 1.
38. **Yates JR, 3rd Link AJ, and Schieltz D.** Direct analysis of proteins in mixtures. Application to protein complexes. *Methods Mol Biol* 146: 17–26, 2000.
39. **Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidlingmaier S, Houfek T, Mitchell T, Miller P, Dean RA, Gerstein M, and Snyder M.** Global analysis of protein activities using proteome chips. *Science* 293: 2101–2105, 2001.
40. **Zhu H, Klemic JF, Chang S, Bertone P, Casamayor A, Klemic KG, Smith D, Gerstein M, Reed MA, and Snyder M.** Analysis of yeast protein kinases using protein chips. *Nat Genet* 26: 283–289, 2000.